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이 학 석 사 학 위 논 문

**^{11}C -(+3)N-Methyl-3-piperidylbenzilate
PET Study of Muscarinic Acetylcholine
Receptor Occupancy by Antimuscarinic
Agents**

항무스카린제에 의한 무스카린성
아세틸콜린 수용체 점유의
 ^{11}C -(+3)N-Methyl-3-
piperidylbenzilate PET 연구

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Abstract

^{11}C -(+3)N-Methyl-3-piperidylbenzilate PET Study of Muscarinic Acetylcholine Receptor Occupancy by Antimuscarinic Agents

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Muscarinic acetylcholine receptor (mAChR) is implicated in various central and peripheral nervous system disorders. The *in vivo* imaging of brain receptors by positron emission tomography (PET) allows the estimation of the precise localization of muscarinic receptors and the pharmacological characterization of the antimuscarinics. ^{11}C -(+3)N-methyl-3-piperidylbenzilate (^{11}C -(+3)-NMPB) is a recently developed PET radioligand, which has preferential binding affinity to the M_1 and M_2 subtypes of mAChR. Additionally, it has more favorable kinetic properties (transient equilibrium).

In the present study, we suggested a new semi-quantification method for simplified preclinical ^{11}C -(+)-3-NMPB PET studies, which we compared to a previous quantification method by investigating changes in mAChR occupancy by antimuscarinic agents. Ninety min dynamic ^{11}C -(+)-3-NMPB PET studies were performed in ICR mice ($n = 22$). The transient equilibrium was assumed on the difference of the time-activity curves between the region of interest and the reference tissue at the vehicle condition ($n = 4$). The binding potential (BP) of ^{11}C -(+)-3-NMPB was estimated using Logan graphical analysis with cerebellar reference tissue input function. The equilibrium area under the curve ratio (AUC ratio) between the target and reference tissue at the equilibrium state after the injection of ^{11}C -(+)-3-NMPB. The percent difference in BP (or occupancy) and AUC ratio in various cortical and subcortical regions between dose conditions (solifenacin [1, 3 and 10 mg/kg, i.v.] or oxybutynin [0.1, 0.3 and 1 mg/kg, i.v.]) were calculated compared to the vehicle. The relationship between the BP and AUC ratio was estimated by correlational analysis. The 50% effective dose (ED_{50}) of the antimuscarinics was calculated from the degree of percentage *in vivo* specific ^{11}C -(+)-3-NMPB binding in brain regions. Finally, a set of statistical tests was performed for comparisons between BP and AUC ratio data. At 30 - 60 min after the injection of ^{11}C -(+)-3-NMPB, radioactivity was shown to transient equilibrium (mean of $t^* = 17.5$ min) at the vehicle condition. ^{11}C -(+)-3-NMPB distribution in the cortical and subcortical regions were well visualized consistently with the known distribution of mAChR. The striatum showed the greatest changes in BP (36.9 - 80.5% after 1, 3, and 10 mg/kg solifenacin and

9.4 – 75.9% after 0.1, 0.3, and 1 mg/kg oxybutynin, respectively). The striatum showed the greatest changes in AUC_{30-60min} ratio (40.9 – 66.1% after 1, 3, and 10 mg/kg solifenacin and 12.4 – 61.3% after 0.1, 0.3, and 1 mg/kg oxybutynin, respectively). BP and AUC_{30-60min} ratio values decreased after the administration of solifenacin ($F(9,36) = 29.2$, $P < 0.01$ and $F(9,36) = 29.4$, $P < 0.01$) or oxybutynin ($F(9,36) = 35.36$, $P < 0.01$ and $F(9,36) = 39.16$, $P < 0.01$) in a dose-dependent manner. Excellent correlations between varying levels of BP and AUC_{30-60min} ratio per solifenacin and oxybutynin doses were found across the regions. ED₅₀ was estimated in the striatum (ED₅₀ = 2.512 mg/kg and 3.162 mg/kg of solifenacin, and ED₅₀ = 0.309 mg/kg and 0.312 mg/kg of oxybutynin, respectively) and the cortex (ED₅₀ = 1.408 mg/kg and 1.092 mg/kg of solifenacin, and ED₅₀ = 0.486 mg/kg and 0.471 mg/kg of oxybutynin, respectively). Data demonstrated that ¹¹C-(+)-3-NMPB PET may be applied to *in vivo* mAChR quantification studies. In this study, the results suggested that semi-quantification of ¹¹C-(+)-3-NMPB binding with the equilibrium AUC ratio method may extend the application of ¹¹C-(+)-3-NMPB PET techniques to assess the efficacy of various mAChR-targeting drugs.

Keywords: PET pharmacokinetics, mAChR occupancy, compartment modeling

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INTRODUCTION

1. Muscarinic acetylcholine receptor (mAChR).

Muscarinic acetylcholine receptor (mAChR) agonism and antagonism are applied in drug development. mAChRs are type of acetylcholine receptor (AChR) with nicotinic acetylcholine receptors (nAChRs). Acetylcholine binding to mAChR is responsible for the activation of an intracellular G-protein (1) as AChR is a part of G-protein-coupled receptors (GPCRs). mAChR mediates various functions of the peripheral and central nervous system (CNS) such as memory, learning, and motor control (2). There are five distinct subtypes, M_1 - M_5 , and each has different roles in various tissues. The striatum has been reported to have the highest levels of cholinergic markers in the brain. mAChRs are associated with a variety of diseases when its function is impaired. Antimuscarinics, such as oxybutynin and solifenacin are used to treat diseases by blocking mAChR. For treatment of overactive bladder (OAB), these agents reduce the frequency of detrusor contractions (3), but they are associated with side effects, such as dry mouth and blurred vision (4). In particular, these agents' side effects concern CNS dysfunction, and cognitive impairment, as they can cross the blood brain barrier (BBB) and bind to mAChR (5). This characteristic makes it possible to study mAChR occupancy in the CNS using PET.

2. PET and radiotracers for investigation of neurotransmitter function

Positron emission tomography (PET) is a device that can quantitatively measure physiological indices in the human body by imaging the *in vivo* distribution of various biochemical substances. PET is widely used to measure biochemical or pathological phenomena, to diagnosis disease, to assist in prognosis after the treatment and establishing a treatment plan. PET is a technique that detects two gamma rays of 511 keV of 180° emitted by a positron when it meets with electrons around the nucleus and disappears. PET acquires images using radiopharmaceuticals labeled with unstable radioactive isotopes, such as ^{18}F , ^{11}C , ^{13}N , and ^{15}O , with a relatively small number of neutrons compared to the number of proton in the nucleus.

^{11}C -(+3)N-methyl-3-piperidylbenzilate (^{11}C -(+3)-NMPB) is a recently developed PET radioligand which has preferential binding affinity to the M_1 and M_2 subtypes of mAChR. The previous radioligands for muscarinic receptors showed low level of uptake to the brain (6) and also slow dissociation rates at the binding sites *in vivo*. These properties limit the measurement of correct receptor density, as equilibrium is not reached within the isotope half-life and scan time (7). In contrast, ^{11}C -(+3)-NMPB has more favorable kinetic properties in evaluating the function of muscarinic receptors because of relatively faster dissociation (8) from receptors than previous radioligands and proper binding affinity. Therefore, we assumed that equilibrium will be easily reached and that the condition of the radiotracer

will be fit to provide a basis for the new parameters we suggested in this study.

^{11}C -(+)-3-NMPB PET for evaluating mAChR function has been previously performed in humans (9), monkeys (6, 10), and small animals (5). However, there have not been sufficient pertinent preclinical and clinical studies. In this present study we provide useful information on the kinetics of radiotracers as well as the preclinical data from ^{11}C -(+)-3-NMPB PET study in mice.

3. PET imaging and quantification for receptor occupancy studies

Receptor imaging using PET is a powerful tool for noninvasively measuring receptor occupancy *in vivo* (11). *In vivo* PET studies on ligand-receptor interactions require quantification of receptors and ligands. However, PET data do not provide direct information on receptor concentration, therefore, mathematical modeling is required (12). The most commonly used PET quantification parameter is the standardized uptake value (SUV) (13). Since this parameter can be semiquantitatively analyzed, it is important to know the arterial input function to determine the input to the tissue for *in vivo* quantification. Depending on the physiological distribution of the drug, the tissue is considered to be divided into multiple compartments. The majority of radioligands do not provide enough information on the arterial input function, but in the reference tissue model, it allows us to estimate nonspecific binding from the reference tissue (14). The reference tissue compartment model is

based on the following equations [1-3] (14, 15),

$$dC_r(t)/dt = K'_1 C_p(t) - k'_2 C_r(t) \quad [1]$$

$$dC_f(t)/dt = K_1 C_p(t) - k_2 C_f(t) - k_3 C_f(t) + k_4 C_b(t) \quad [2]$$

$$dC_b(t)/dt = k_3 C_f(t) - k_4 C_b(t), \quad [3]$$

where C_p is the metabolic corrected plasma concentration ($\text{kBq} \cdot \text{ml}^{-1}$), C_r is the concentration in the reference tissue ($\text{kBq} \cdot \text{ml}^{-1}$), C_f is the concentration of the free ligand ($\text{kBq} \cdot \text{ml}^{-1}$), C_b is the concentration of specifically bound ligand ($\text{kBq} \cdot \text{ml}^{-1}$), K_1 is the rate constant for transfer from plasma to free compartment ($\text{ml} \cdot \text{ml}^{-1} \cdot \text{min}^{-1}$), k_2 is the rate constant for transfer from free to plasma compartment (min^{-1}), k_3 is the rate constant for transfer from free to bound compartment (min^{-1}), and t is time (min).

3-1. mAChR quantification using compartment modeling, binding potential

The binding potential (BP) is a fundamental measure to calculate the receptor occupancy *in vivo*. BP is a parameter that indicates the density of receptors in the target regions and the affinity of radioligands to the individual receptors. The BP is the gold standard method of quantification of receptor occupancy (16). This method eliminates the need for blood sampling using the simplified reference tissue compartment model (SRTM) or the Logan plot with the

reference tissue input function (17).

3-2. mAChR quantification using non-compartment modeling, transient equilibrium, and AUC ratio

In pharmacokinetics, the area under the curve (AUC) reflects the exposure after administration of a dose of the drug in the tissue. AUC is used to determine the bioavailability of a given amount of a drug. In PET pharmacokinetics, AUC means the uptake of the radioactivity in the tissue. In the receptor occupancy study, the AUC method can only be employed for the equilibrium state (transient equilibrium). Equilibrium approaches can complement the disadvantages of PET data that does not provide sufficient information to distinguish bound and free regions. The equilibrium approaches were introduced to simplify the model because it can show the bound and free constant directly. In the neuroreceptor binding studies, the transient equilibrium means the uptake and clearance rate constant of radioligands are the same for all simulations. The equilibrium analysis has previously been applied in a variety of studies, such as the study of D2-dopamine receptor density in humans with schizophrenia (18, 19).

Simplification of the quantification method is important in terms of time and cost savings. The reference tissue method reduces the

complexity of the data analysis procedures as it does not require arterial cannulation and blood sampling (14). Likewise, the AUC ratio would also simplify the experimental procedure as it is not dependent on compartment modeling and it may reduce scan time in contrast to BP. The AUC ratio can be calculated by the ratio of the radioactivity of the cerebellum. This is possible because ^{11}C -(+)-3-NMPB has the property of easily reaching equilibrium. The present study investigated the validity and application of the AUC ratio, a more simplified quantification method in transient equilibrium, by applying it to the ^{11}C -(+)-3-NMPB PET study.

4. Research objectives

A. mAChR visualization and its' quantification in small animals

By utilizing the pharmacokinetic properties of ^{11}C -(+)-3-NMPB, we quantified muscarinic receptors distribution in the CNS using PET and the antimuscarinics.

B. mAChR quantification simplified by using the transient equilibrium of the radioligand

We evaluated a semi-quantification method, the AUC ratio, optimized for simplified preclinical ^{11}C -(+)-3-NMPB PET studies.

C. Validation of the newly developed method in terms of application

We compared the two methods, BP and AUC ratio, and expanded their application.

MATERIALS AND METHODS

Animals

Twenty two ICR mice weighing 20 - 25 g (6 - 7 weeks old; male) were used in this study. We received approval about the protocols concerning the experimental animals from the Seoul National University Bundang Hospital Institutional Animal Care and Use Committee (SNUBH IACUC No. BA1606-202/031-01). All animal care was conducted in the preclinical center of SNUBH and the mice were housed in plastic cages with corn-top and aspen bedding. All animals were allowed access to purified water and experimental animal feed (Purinafeed, Cargill Agril Purina, Inc., Gyeonggi, Korea) until they were used for in the experiments. Animal rooms were maintained at constant temperature (21 – 24°C), relative humidity (45 - 60%), and photoperiod (12 h light/day).

Preparation of drugs

Solifenacin (HalleChem Pharma Co., Ltd., Chongqing, China) and Oxybutynin (Nanjing Chemlin Chemical Industry Co., Ltd., Nanjing, China) were dissolved in saline and administered i.v. (injection volume : 100 µl). Animals received an injection of solifenacin (0, 1, 3, and 10 mg/kg) or oxybutynin (0, 0.1, 0.3, and 1 mg/kg) (5).

Radiotracers

^{11}C -(+)-3-NMPB was synthesized from desmethyl-(+)-3-NMPB by ^{11}C -methylation reaction using ^{11}C -methyl iodide in the TRACERlab FX C pro module (GE Healthcare, Milwaukee, MI) with little modification, according to the previously reported methods (20). Briefly, ^{11}C -carbon dioxide was produced via the $^{14}\text{N}(\text{p},\alpha)^{11}\text{C}$ nuclear reaction by bombardment of nitrogen gas (99.9999%) with a 13 MeV proton beam produced by cyclotron (KOTRON-13 installed at Seoul National University Bundang Hospital) and transferred to TRACERlab FX C pro module. The ^{11}C -carbon dioxide is converted to ^{11}C -methane for further gas phase reaction to make ^{11}C -methyl iodide. ^{11}C -Methyl iodide was obtained from ^{11}C -methane on-line by gas-phase conversion with iodine. ^{11}C -Methyl iodide carried in a flow of helium gas (30 mL/min) was bubbled into a solution of N,N-dimethylformamide (0.4 mL) containing 1 mg of desmethyl-(+)-3-NMPB at -20°C . When radioactivity had peaked in the reactor, the solution was heated to 80°C and maintained for 5 min. After cooling, the reaction mixture was quenched by addition of a high-performance liquid chromatography (HPLC) solvent (1.2 mL) and injected into a reverse-phase HPLC system. The HPLC purification (column: Xterra RP-18, 10 x 250 mm) using an ultraviolet detector (254 nm) and a gamma-ray detector was performed in 30% $\text{CH}_3\text{CN}/0.1\text{ M}$ sodium acetate/0.1% AcOH at a flow rate of 3 mL/min. The collected solution around 16 min of retention time was exchanged with 6 to 8%

ethanol–saline solution using a tC18 Sep-Pak cartridge to remove the HPLC solvent. The identity was confirmed by coinjection with authentic compound, NMPB, using an analytical HPLC system (Xterra RP-18, 4.6 x 250 mm, 10 to 90% CH₃CN/0.1 M sodium acetate/0.1% AcOH (0 to 15 min), 1.2 mL/min).

PET data acquisition

PET studies were performed with a NanoPET/CT system (Mediso Inc., Budapest, Hungary) using the dynamic mode for data acquisition. ICR mice were anesthetized with 1.5-2% isoflurane (2 L/min flow rate). After induction of anesthesia, a catheter was inserted into the tail vein for intravenous injection of the antimuscarinics and ¹¹C-NMPB. At 10 min after the injection of each agent, ¹¹C-(+)-3-NMPB (4 to 25 MBq) was injected intravenously. Ninety-minute dynamic PET scan was performed (12 × 10 sec, 16 × 30 sec, 8 × 1 min, 18 × 4 min).

PET data analysis

The volumes of interest (VOIs) were placed on the striatum, cortex, hippocampus, thalamus, and midbrain and the cerebellum was set as the reference region. VOIs were defined using an automated anatomical labeling template embedded in pixel-wise kinetic modeling function of software (PMOD version 3.6, PMOD Technologies, Zurich, Switzerland). The obtained time-activity curves in the VOIs were fitted to a simplified reference tissue model using the PMOD software. Radioactivity measured in tissues was converted to the SUV by the formula [5],

$$SUV = \frac{C_T}{ID} \cdot w, \quad [5]$$

where C_T ($\mu\text{Ci}/\text{ml}$) = activity at a pixel within the tissue defined by an VOI, ID (μCi) = injected dose, and w (kg) = animals body weight. The equilibrium in time activity curves was analyzed by linear regression analysis using Prism (version 5; GraphPad Software, Inc., San Diego, CA).

1. Binding Potential (BP)

The binding potential (BP) of ^{11}C -(+)-3-NMPB for muscarinic receptors in ROIs was calculated. The BP of ^{11}C -(+)-3-NMPB obtained during this time was calculated by the equation [6],

$$BP = (C_T - C_{ND})/C_{ND} , \quad [6]$$

where, C_{ND} ($\mu\text{Ci/ml}$) = activity at the reference region.

The simplified reference tissue model (SRTM) method was used to calculate the BP. The calculation of the change (%) of BP in two groups according to drug condition [7],

$$\Delta BP = \frac{(BP_{treatment} - BP_{baseline})}{(BP_{treatment})} \cdot 100\% , \quad [7]$$

where, $BP_{treatment}$ = BP after drug treatment, $BP_{baseline}$ = BP in the vehicle condition.

2. The ratio of area under the curve (AUC ratio)

The area under the curve was defined by integral of the time-activity curve of y = radioactivity and the x = time from the equilibrium state post-injection of ^{11}C (+)-3-NMPB. The ratio of the area under the curve (AUC ratio) was defined as the ratio of the AUC of the target

region divided by the AUC of the cerebellum. The change (%) of AUC ratio in two groups was calculated according to drug condition [8],

$$\Delta \text{AUC ratio} = \frac{(AUC_{\text{treatment ratio}} - AUC_{\text{baseline ratio}})}{(AUC_{\text{treatment ratio}})} \cdot 100\%, \quad [8]$$

where, $AUC_{\text{treatment ratio}}$ = AUC ratio after drug treatment, $AUC_{\text{baseline ratio}}$ = AUC ratio in the vehicle condition.

Method validation and Statistical analysis

Statistical differences of BP and AUC ratio between the drug administration conditions were tested by two-way ANOVA with Bonferroni post-hoc analysis. $P < 0.05$ was considered statistically significant. All error bars in the statistical analysis are standard errors of the mean (SEM). The correlation tests between BP and AUC ratio values were analyzed using Prism (version 5; GraphPad Software, Inc.). The 50% effective dose (ED_{50}) of the antimuscarinics in two parameters was analyzed by a non-linear regression model embedded in Prism from the change of BP and AUC ratio.

RESULTS AND DISCUSSION

In the present study, we performed a preclinical mAChR occupancy study by using ^{11}C -(+)-3-NMPB PET. In addition, we validated and applied the AUC ratio method based on the kinetic properties of ^{11}C -(+)-3-NMPB.

The selective radiotracer for muscarinic cholinergic receptor, ^{11}C -(+)-3-NMPB, was efficiently and rapidly synthesized from desmethyl-(+)-3-NMPB by ^{11}C -methylation reaction using ^{11}C -methyl iodide in the TRACERlab FX C pro module (GE Healthcare, Milwaukee, MI). After ^{11}C -methylation, ^{11}C -(+)-3-NMPB was purified in reverse-phase preparative-HPLC system and the desired product, ^{11}C -(+)-3-NMPB, was collected at approximately 16 min of retention time (Figure 1). The solution was exchanged to 6 to 8% ethanol–saline using a tC18 Sep-Pak cartridge to create the biologically injectable solution. The formulated radiotracer displayed no radiolysis for at least 60 min post-formulation without an additional stabilizing agent. Co-injection of the radioactive product with the authentic standard of (+)-3-NMPB under different conditions further established the identity of ^{11}C -(+)-3-NMPB (Figure 2). Consequently, the radiochemical yield was $5.8 \pm 0.8\%$ ($n = 35$, non-decay corrected) with over 99% radiochemical purity. Total elapsed time was 45 ± 1 min and the specific activity of ^{11}C -(+)-3-NMPB at the end of the synthesis was 67 ± 13 GBq/ μmol .

Radioactivity was shown to reach transient equilibrium (mean of $t^* = 17.5$

min) at the vehicle condition in all brain regions (Figure 3). The uptake of the cerebellum, considered as the nondisplaceable binding, was subtracted from the ROI and the specific binding was considered. The highest binding was in the striatum and was followed, in descending order, by the hippocampus, thalamus, cortex, and midbrain. This results indicate that ^{11}C -(+)-3-NMPB has preferential binding affinity to the M_1 and M_2 subtypes of mAChR. ^{11}C -(+)-3-NMPB distribution showed consistent patterns in the brain regions where M_1 and M_2 subtypes of mAChR were present. The subgraph of Figure 3 is the linear regression analysis that determined the equilibrium of the time activity curves in all regions. We were able to determine the equilibrium between 30 - 60 minutes and under bases of linear regression analysis ($P < 0.0001$), and we devised the $\text{AUC}_{30-60\text{min}}$ ratio method. The transient equilibrium indicates that specific binding radioactivity is maximized (21). Therefore, *in vivo* specific binding of ^{11}C -(+)-3-NMPB was sufficiently maximized between 30 - 60 min, and the cerebellum as reference tissue was assumed to be of free ligand concentration. In addition, the previous mAChR radioligands have a slow dissociation rate from the mAChR (7), making it difficult to ascertain the true equilibrium state during the scan time. On the other hand, ^{11}C -(+)-3-NMPB seems to reach equilibrium rapidly. It is believed that they have more favorable kinetic properties to estimate the mAChR occupancy study.

After the administration of the antimuscarinics, solifenacin and oxybutynin, we confirmed the properties of ^{11}C -(+)-3-NMPB via receptor occupancy studies. First, we conducted a study using BP as the gold standard method of

receptor occupancy. BP was used to quantify cerebral mAChR occupancy in each brain region by the antimuscarinics using ^{11}C -(+)-3-NMPB PET (Figure 4). With competitive binding of ^{11}C -(+)-3-NMPB and the antimuscarinics, we confirmed the receptor occupancy of the drugs. After the i.v. injection of solifenacin (1.0 - 10.0 mg/kg), the distribution of ^{11}C -(+)-3-NMPB was significantly decreased in the striatum (36.9 - 80.4%), cortex (29.9 - 75.1%), hippocampus (38.4 - 80.4%), thalamus (41.1 - 73.6%), and midbrain (34.5 - 73.5%), in a dose-dependent manner. In addition to the i.v. injection of oxybutynin (0.1 - 1.0 mg/kg), the distribution of ^{11}C -(+)-3-NMPB was significantly decreased in the striatum (9.4 - 75.9%), cortex (-6.6 - 69.2%), hippocampus (7.4 - 70.0%), thalamus (11.9 - 69.4%), and midbrain (-3.9 - 59.2%), in a dose-dependent manner (Table 1). Antimuscarinics bound to the brain regions crossing the BBB and the number of mAChRs blocked by the drugs could be quantified by ^{11}C -(+)-3-NMPB. We confirmed that ^{11}C -(+)-3-NMPB preferentially bound to mAChR.

We, then, proceeded with an occupancy study to compare the $\text{AUC}_{30-60\text{min}}$ ratio, a new parameter proposed in this study, to BP (Figure 5). After the i.v. injection of solifenacin (1.0 - 10.0 mg/kg), the distribution of ^{11}C -(+)-3-NMPB was significantly decreased in the striatum (48.8 - 60.5%), cortex (41.9 - 51.3%), hippocampus (45.9 - 58.7%), thalamus (43.8 - 56.8%), and midbrain (33.7 - 57.1%), in a dose-dependent manner. In addition to the i.v. injection of oxybutynin (0.1 - 1.0 mg/kg), the distribution of ^{11}C -(+)-3-NMPB was significantly decreased in the striatum (6.1 - 62.6%), cortex (-3.2 - 50.9%),

hippocampus (9.7 - 52.9%), thalamus (12.6 - 50.1%), and midbrain (5.6 - 42.2%), in a dose-dependent manner (Table 2). In some subjects, however, the decreased rate occurred when they were administered with a low dose. It was assumed that there was no difference from the vehicle condition because the drug dose was very low.

The striatum showed the greatest changes in BP and AUC_{30-60min} ratio values after the administration of solifenacin ($F(9, 36) = 29.2, P < 0.01$ and $F(9, 36) = 29.4, P < 0.01$) or oxybutynin ($F(9, 36) = 35.36, P < 0.01$ and $F(9, 36) = 39.16, P < 0.01$). As a result of quantification with two parameters, similar results were obtained for all brain regions. Therefore, we obtained evidence suggesting that the AUC_{30-60min} ratio is a new simplified parameter. Although there are differences in areas where the second most frequent change occurred, the difference can be neglected by about 0.01%, therefore, it can be concluded that the two parameters produced comparable results.

A correlation test was performed to validate the AUC_{30-60min} ratio method (Figure 6). The correlation between ¹¹C-(+)-3-NMPB specific binding of two parameters in the striatal and cortical areas was investigated in a dose-dependent manner. The correlations across the regions were excellent (Table 3). The non-linear regression analysis results between two values were $R^2 = 0.97, P < 0.0001$ for the striatum and $R^2 = 0.96, P < 0.0001$ for the cortex with solifenacin and $R^2 = 0.87, P < 0.0001$ for the striatum and $R^2 = 0.81, P < 0.0001$ for the cortex with oxybutynin. Based on these results, we can suggest that the AUC_{30-60min} ratio may be a new parameter sufficient to replace BP.

We suggest that the $AUC_{30-60min}$ is a simpler method as the $AUC_{30-60min}$ requires only 30 - 60 min of scan time with transient equilibrium, while BP requires 90 min of scan time. In addition, BP requires a complex analysis procedure as it involves mathematical modeling, but the $AUC_{30-60min}$ ratio is technically fast and simple, as it does not require any modeling procedures.

The ED_{50} of the antimuscarinics was calculated to broaden the application range of the $AUC_{30-60min}$ ratio parameters. We applied it to assess the ED_{50} which is used to determine drug dose and toxicity. The results of the ED_{50} generated similar values for both parameters. ED_{50} was estimated in the striatum ($ED_{50} = 2.512$ mg/kg and 3.162 mg/kg with solifenacin, and $ED_{50} = 0.309$ mg/kg and 0.312 mg/kg with oxybutynin, respectively) and the cortex ($ED_{50} = 1.408$ mg/kg and 1.092 mg/kg with solifenacin, and $ED_{50} = 0.486$ mg/kg and 0.471 mg/kg with oxybutynin, respectively) (Figure 7).

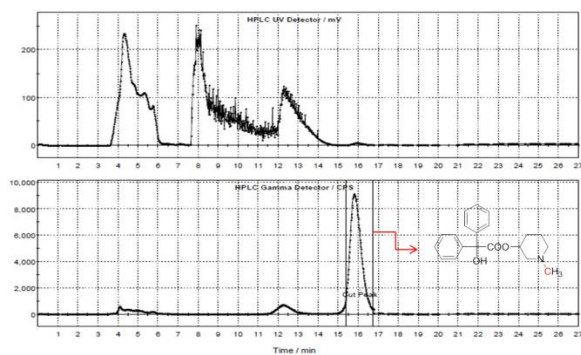


Figure 1. The HPLC chromatogram of the reaction mixture (upper: UV-254 nm, bottom: gamma-ray).

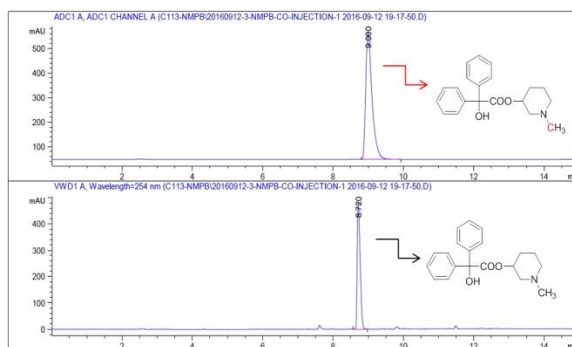


Figure 2. The HPLC chromatogram of the coinjection with authentic compound (upper: gamma-ray, bottom: UV-254 nm).

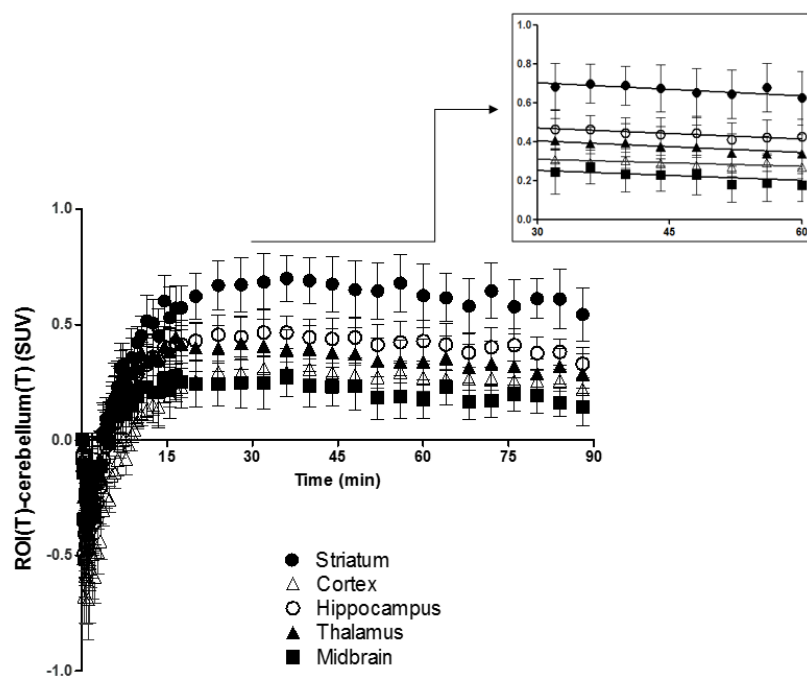


Figure 3. Time activity curves of ^{11}C -(+)-3-NMPB at the vehicle condition in the striatum (black circles), cortex (white triangles), hippocampus (white circles), thalamus (black triangles), and midbrain (black squares). The subgraph indicates the result of the linear regression analysis within 30 - 60 min after the injection of ^{11}C -(+)-3-NMPB (up right).

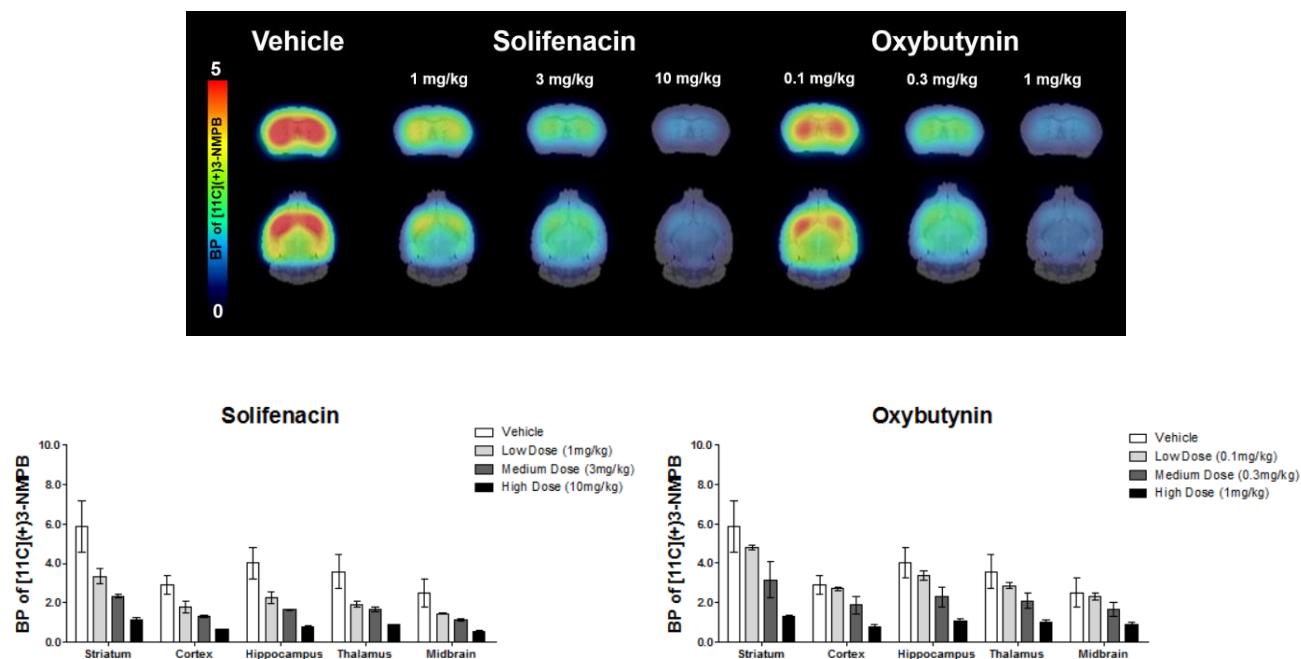


Figure 4. Representative BP parametric PET images in the brain regions that received ^{11}C -(+)-3-NMPB after the administration of solifenacin or oxybutynin (upper). Effects of i.v. injection of solifenacin and oxybutynin on the BP of ^{11}C -(+)-3-NMPB in the striatum, cortex, hippocampus, thalamus, and midbrain (bottom).

Table 1. The changes in BP of ^{11}C -(+)-3-NMPB in the VOIs after the i.v. injection of antimuscarinics.

Drug	Control					Solifenacin						Oxybutynin								
Dose	Vehicle		1 mg/kg			3 mg/kg			10 mg/kg			0.1 mg/kg			0.3 mg/kg			1 mg/kg		
Region	BP mean	SEM	BP mean	SEM	p	BP mean	SEM	p	BP mean	SEM	p	BP mean	SEM	p	BP mean	SEM	p	BP mean	SEM	p
Striatum	5.8608	1.2860	3.3393	0.3704	0.0065	2.3338	0.0690	0.0001	1.1003	0.1217	0.0001	4.7992	0.1432	0.4596	3.1446	0.9226	0.0070	1.2779	0.0915	0.0001
	2.8918	0.4620	1.7806	0.2758	0.3583	1.3274	0.0623	0.1269	0.6352	0.0198	0.0162	2.7056	0.0882	0.9926	1.8836	0.4495	0.5001	0.7833	0.0798	0.0439
Hippocampus	4.0094	0.7781	2.2436	0.2789	0.0736	1.6362	0.0338	0.0109	0.7536	0.0543	0.0004	3.3746	0.2182	0.7958	2.2820	0.5129	0.1184	1.0913	0.1115	0.0036
Thalamus	3.5811	0.8502	1.9197	0.1630	0.0982	1.6579	0.1325	0.0465	0.8605	0.0321	0.0032	2.8729	0.1560	0.7403	2.0907	0.3750	0.2034	0.9992	0.1204	0.0107
Midbrain	2.4902	0.7372	1.4501	0.0351	0.4107	1.1413	0.0473	0.2151	0.5443	0.0242	0.0435	2.2983	0.1763	0.9919	1.6445	0.3606	0.6301	0.9020	0.0869	0.1639

The values of BP obtained from 4 mice in the vehicle and 3 mice in the antimuscarinics .

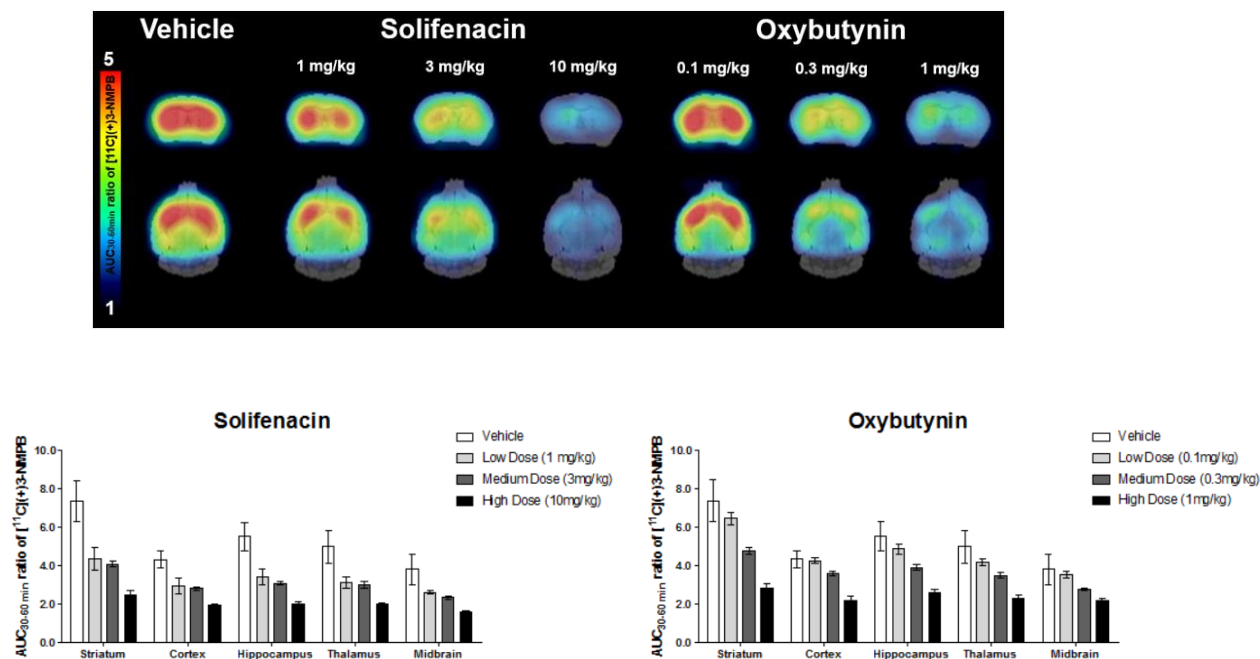


Figure 5. Representative PET images of the brain regions that received ¹¹C-(+)-3-NMPB after the administration of solifenacin or oxybutynin (upper). Effects of i.v. injection of solifenacin and oxybutynin on the AUC_{30-60min} ratio of ¹¹C-(+)-3-NMPB in the striatum, cortex, hippocampus, thalamus and midbrain (bottom).

Table 2. The changes in AUC_{30-60min} ratio of ¹¹C-(+)-3-NMPB in the VOIs after the i.v. injection of antimuscarinics.

Drug	Control					Solifenacin						Oxybutynin								
Dose	Vehicle		1 mg/kg			3 mg/kg			10 mg/kg			0.1 mg/kg			0.3 mg/kg			1 mg/kg		
Region	AUC ratio mean	SEM	AUC ratio mean	SEM	p	AUC ratio mean	SEM	p	AUC ratio mean	SEM	p	AUC ratio mean	SEM	p	AUC ratio mean	SEM	p	AUC ratio mean	SEM	p
Striatum	7.3669	1.0673	4.3558	0.5821	0.0008	4.0876	0.1561	0.0003	2.4943	0.2094	0.0001	6.4509	0.3108	0.4673	4.7672	0.1875	0.0028	2.8484	0.2153	0.0001
Cortex	4.3217	0.4182	2.9227	0.4108	0.1747	2.8055	0.0770	0.1293	1.9510	0.0837	0.0088	4.2463	0.1576	0.9992	3.5989	0.1143	0.6432	2.1983	0.1917	0.0168
Hippocampus	5.5101	0.7511	3.4008	0.4170	0.0218	3.0810	0.0798	0.0072	1.9940	0.1406	0.0001	4.8661	0.2751	0.7160	3.8979	0.1352	0.0872	2.5709	0.1864	0.0007
Thalamus	4.9772	0.8661	3.1012	0.3016	0.0461	2.9868	0.1655	0.0321	2.0002	0.0758	0.0009	4.1800	0.1844	0.5738	3.4857	0.1421	0.1228	2.3195	0.1438	0.0023
Midbrain	3.8056	0.7912	2.6211	0.0972	0.2887	2.3362	0.0792	0.1462	1.5913	0.0394	0.0153	3.5178	0.1859	0.9617	2.7442	0.0570	0.3506	2.2008	0.1249	0.0892

The values of AUC_{30-60min} ratio obtained from 4 mice in the vehicle and 3 mice in the antimuscarinics.

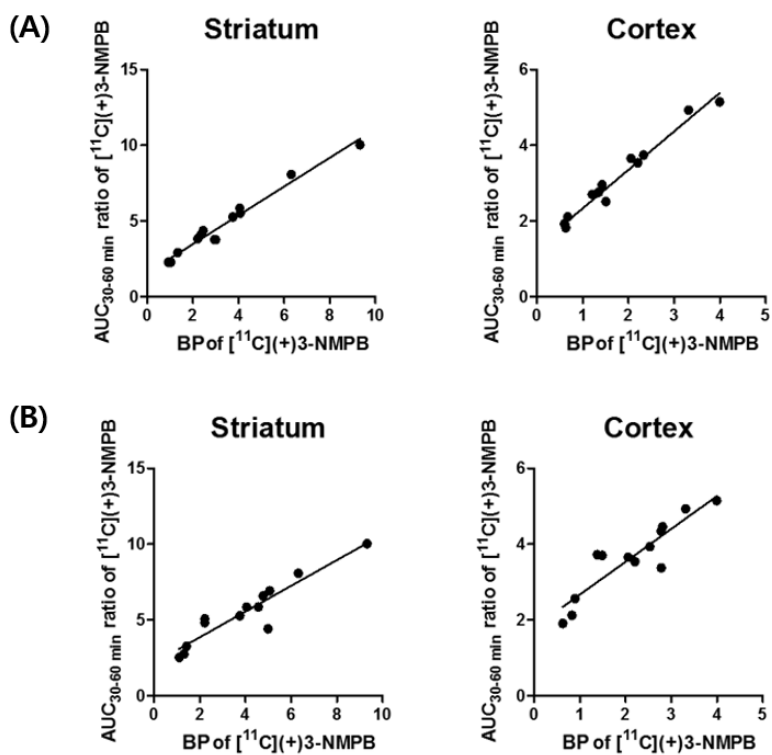


Figure 6. Correlation between BP (X-axis) and the $\text{AUC}_{30-60 \text{ min}}$ ratio (Y-axis) determined using $^{11}\text{C}-(+)\text{-3-NMPB}$ in solifenacin (A) and oxybutynin (B) in the striatum and cortex.

Table 3. Correlation statistics between BP and AUC_{30-60min} ratio.

Drugs	Region	<i>P</i>	<i>Df</i>	<i>R</i>²
Solifenacin	Striatum	<0.0001	11	0.97
	Cortex	<0.0001	11	0.96
Oxybutynin	Striatum	<0.0001	11	0.87
	Cortex	<0.0001	11	0.81

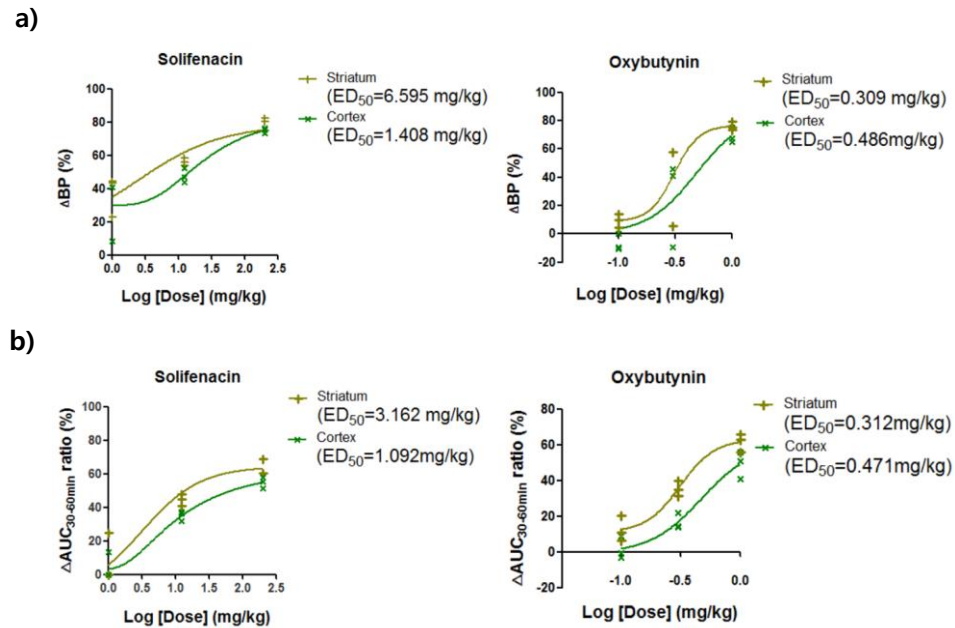


Figure 7. Dose-BP curves (a) and the dose-AUC_{30-60min} ratio curves (b) in the striatum (+) and the cortex (x) of ICR mice after i.v. injection of solifenacin ($n = 9$) and oxybutynin ($n = 9$). The muscarinic receptor occupancy was determined from the degree of reduction by antimuscarinic agents of *in vivo* specific ¹¹C-(+3)-NMPB binding.

CONCLUSION

Our data demonstrated that the receptor occupancy study of mAChR with ^{11}C -(+)-3-NMPB PET is a powerful tool for measuring the pharmacological effects of the antimuscarinics. We confirmed that ^{11}C -(+)-3-NMPB has characteristics that bear favorable properties for mAChR occupancy evaluation. In addition, we demonstrated a means of simplification for *in vivo* PET quantification compared to conventional parameters. To this end, the $\text{AUC}_{30-60\text{min}}$ ratio methods may efficiently serve in mAChR-occupancy studies with ^{11}C -(+)-3-NMPB PET. However, the ED_{50} of the antimuscarinics estimated in this study should be validated for clarifying the drug-response. There have been few preclinical ^{11}C -(+)-3-NMPB PET studies. Therefore, our data may serve as a basis for expansion into clinical trial studies on mAChR-targeting drugs. In addition, the AUC ratio parameter can be used to simplify the procedures employed in these studies.

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국문 초록

항무스카린제에 의한 무스카린성 아세틸콜린 수용체 점유의 ^{11}C -(+3)N-Methyl-3- piperidylbenzilate PET 연구

무스카린성 아세틸콜린 수용체 (mAChR)는 다양한 신경계 질환에 연관되어 있다. 양전자 방출 단층 촬영 (PET)에 의한 생체 이미징은 대뇌 무스카린 수용체의 정확한 국소화 및 약리학적 특성 평가를 가능하게 한다. 최근에 개발된 ^{11}C -(+3)N-methyl-3-piperidylbenzilate (^{11}C -(+)-3-NMPB)는 mAChR의 M1과 M2 아형에 우선적인 결합 친화력을 갖는 PET 방사성리간드이며 전임상 연구에서 이전의 방사성리간드보다 유리한 약동학적 특성을 가져서 조기에 transient equilibrium에 도달하는 것으로 보고되었다. 본 연구에서는 전임상 ^{11}C -(+)-3-NMPB PET 연구의 단순화를 위해 transient equilibrium에서 평가되는 새로운 semi-quantification 방법을 제시하고 항무스카린제에 의한 mAChR 점유율 변화를 기존 방법론과 비교 평가하였다. **재료 및 방법:** ICR 마우스 ($n = 22$)에

서 90분 동안 동적 ^{11}C -(+)-3-NMPB PET 영상을 획득하였다. Transient equilibrium은 대조군 조건 ($n = 4$) 에서 관심 조직과 참고 조직의 시간-방사성농도 곡선을 통해 평가하였다. 다양한 대뇌 피질 및 피질 하부 영역에서의 수용체 점유율 (BP)은 소뇌 (cerebellum)를 참고 조직으로 한 Logan 그래프 분석법으로 획득하였다. ^{11}C -(+)-3-NMPB투여 후 평형 상태가 나타나는 지점에서 관심 조직과 참고 조직 간의 곡선하면적 비 (AUC ratio)를 계산하였다. 약물용량별 BP 및 AUC ratio의 대조군 대비 변화율을 계산한 후 통계 검증을 수행하였다. 상관분석을 통해 BP와 AUC ratio 두 변수간의 상관관계를 평가하였다. 뇌 영역에서의 항무스카린제의 반수유효투여량 (ED_{50})을 비선형 회귀 분석에 의해 추정 하였다. **결과:** 대조군 조건에서 ^{11}C -(+)-3-NMPB 투여 후 30-60 분에 transient equilibrium (평균 $t^* = 17.5$ 분)이 관찰되었다. 대뇌 전반에 걸쳐 ^{11}C -(+)-3-NMPB의 분포가 기존에 알려진 대뇌 mAChR의 분포와 일관되게 관찰되었다. 선조체 (striatum)에서 항무스카린제 투여 후 가장 큰 BP의 변화가 관찰되었다 (solifenacin 1 - 10 mg/kg 투여 후 36.9 - 80.5% 그리고 oxybutynin 0.1 - 1 mg/kg 투여 후 9.4 - 75.9%). $\text{AUC}_{30-60\text{min}}$ ratio도 선조체에서 가장 큰 변화를 보였다 (solifenacin 1 - 10 mg/kg 투여 후 40.9 - 66.1% 그리고 oxybutynin 0.1 - 1 mg/kg 투여 후 12.4 - 61.3 %). BP와 $\text{AUC}_{30-60\text{min}}$ ratio 값 모두 solifenacin ($F(9,36) =$

29.2, $P < 0.01$ and $F(9,36) = 29.4$, $P < 0.01$) 및 oxybutynin ($F(9,36) = 35.36$, $P < 0.01$ and $F(9,36) = 39.16$, $P < 0.01$)의 투여 후 용량 의존적으로 감소하였다. 항무스카린제의 투여용량별 BP와 $AUC_{30-60min}$ ratio의 변화 수준 사이의 우수한 상관관계가 대뇌 전 영역에서 나타났다. ED_{50} 는 BP와 $AUC_{30-60min}$ ratio의 대조군 대비 변화율로부터 선조체 (solifenacin $ED_{50} = 2.512$ mg/kg 및 3.162 mg/kg, 및 oxybutynin $ED_{50} = 0.309$ mg/kg 및 0.312 mg/kg)와 대뇌 피질 (solifenacin $ED_{50} = 1.408$ mg/kg 및 1.092 mg/kg, 및 oxybutynin $ED_{50} = 0.486$ mg/kg 및 0.471 mg/kg)에서 평가되었다. **논의:** 본 연구를 통해 우리는 $^{11}C-(+)-3-NMPB$ PET을 생체 내 mAChR 정량화 연구에 적용할 수 있음을 증명하였다. 우리가 제시한 transient equilibrium 상에서 획득한 AUC ratio를 통한 $^{11}C-(+)-3-NMPB$ 의 mAChR 점유율의 semi-quantification 방법은 다양한 mAChR 표적약물의 효능을 평가하기 위한 $^{11}C-(+)-3-NMPB$ PET 연구의 기술 적용범위를 확대할 수 있음을 시사할 수 있다.

중심단어: PET 약동학, 무스카린성 아세틸콜린 수용체(mAChR) 점유, 구획모형 모델링

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